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(54) Title: HA-2 ANTIGENIC PEPTIDE

(57) Abstract

The present invention discloses the first peptide sequence of a so-called minor H antigen. The minor H antigens are associated with the Graft versus Host Disease. The peptide and its derivatives find many uses in bone marrow transplantation, organ transplantation and in the treatment of leukemia. The peptide and its derivatives can be incorporated in vaccines, in pharmaceutical formulations and they can be used in diagnostic test kits. The peptide is derived from the HA-2 minor antigen and has the sequence YXGEVXVSV, wherein X represents a leucine or an isoleucine residue. Both donors and recipients in bone marrow transplantation can be treated with the peptides, optionally in combination with other peptides, coupled to carriers, with suitable excipients and/or adjuvants.

Title: HA-2 antigenic peptide

This invention relates to the field of immunology,
especially cellular immunology. It particularly relates to the
area of transplantation of organs, tissues or cells
(especially bone marrow) and possible immunological reactions
5   caused by such transplantations.

Bone marrow transplantation (BMT) finds its clinical
application in the treatment of, for instance, severe aplastic
anaemia, leukaemia and immune deficiency disease.

In the early days many of these transplantations resulted
10   in rejection of the transplant or in Graft versus Host Disease
(GvHD). It is nowadays clear that these effects are largely
due to the presence of major H antigens which function as a
major transplantation barrier. Consequently, improved success
in bone marrow transplantation was reported when matching for
15   the HLA antigens was taken into account. Nowadays, mainly HLA-
identical siblings or HLA-matched unrelated individuals are
used as a source for the donor material. Still, despite the
improvements in HLA-matching, as well as improvements in
pretransplantation chemotherapy and/or radiotherapy and the
20   use of potent immunosuppressive drugs as prophylaxis, as well
as better antibiotics and better isolation techniques for the
donor material, about 20-70% (depending on their age) of the
recipients still suffer from GvHD. In GvHD immunocompetent
donor T cells react against the host tissues. Therefore
25   donation of marrow from which the mature T cells have been

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removed has become a frequently used regimen. However, this often leads to graft rejection or failure, as well as to recurrence of the original disease, which is particularly dramatic in leukaemia.

5   The problems still associated with (particularly) human transplantation can hardly be attributed to the major H antigens, since the donor and recipient are routinely screened for HLA identity. Therefor GvHD may be caused by the disparity of the products of the so called 'minor' H systems (mHag),

10   i.e. Histocompatibility antigens other than those encoded by the MHC.

mHag's have been originally discovered in tumour- and skin rejection studies between congeneic strains of mice. Over 40 mHag loci have been defined, dispersed over the genome, but

15   estimations predict the existence of several hundred loci. One of the better known minor H antigens is the HY antigen.

Several reports have demonstrated the presence of anti-host mHag specific CTL in patients suffering from GvHD after HLA genotypically identical BMT (1-7). In our laboratory, much

20   effort was put into the further characterization of a (small) number of anti-host mHag specific CTLs. Hereto, CTL clones specific for host mHag were isolated from the peripheral blood (PBL) of patients suffering from severe GvHD (8).

Subsequent immunogenetic analyses revealed that the CTL

25   clones (as described above) identified five non-sexlinked mHag, designated HA-1, -2, -3, -4, -5, which are recognized in classical MHC restricted fashion (8). mHag HA-3 is recognized in the presence of HLA-A1 and mHag HA-1, -2, -4 and -5 require

the presence of HLA-A2. Segregation studies demonstrated that each of mHag HA-1 to HA-5 is the product of a single gene segregating in a Mendelian fashion and that HA-1 and HA-2 are not coded within the HLA region (9). The mHag differ from each

5   other in phenotype frequencies; mHag HA-2 appeared very frequent (i.e. 95%) in the HLA-A2 positive healthy population (10).

With regard to the mHag expressed on different tissues, we observed ubiquitous versus restricted tissue distribution

10   of the mHag analysed (11). The expression of the mHag HA-2 is restricted to the cells of the haematopoietic cell lineage, such as thymocytes, peripheral blood lymphocytes, B cells and/or monocytes. Also the bone marrow derived professional antigen presenting cells; the dendritic cells and the

15   epidermal Langerhans cells express the mHag HA-2 (11, 12). The mHag HA-2 is also expressed on haematopoietic stem cells (13), on clonogenic leukemic precursor cells (14), as well as on freshly isolated myeloid and lymphoid leukemic cells (15).

To substantiate the importance of the human mH antigenic

20   systems, we investigated whether the mHag are conserved in evolution between human and non human primates. Therefor, cells from non human primates were transfected with the human HLA-A2.1 gene. Subsequent analyses with our human allo HLA-A2.1 and four mHag HLA-2.1 restricted CTL clones revealed

25   the presentation of ape and monkey allo and mHag HY, HA-1 and HA-2 peptides in the context of the transfected human HLA-A2.1 molecule by ape and monkey target cells. Furthermore, peptides were eluted from HLA-A2.1 molecules expressed on the

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transfected ape cells. An HA-2 positive fraction was identified that showed the same behaviour on reverse phase HPLC as the HA-2 fraction derived from human EBV-LCL. This implicates that the HA-2 peptide is conserved for at least

5   35 million years (16).

A prospective study was performed in order to document the effect of mHag in HLA genotypically identical BMT on the occurrence of acute (grade $\geq$ 2) GvHD. The results of the mHag typing using the CTL clones specific for five well defined

10   mHag HA-1 to HA-5 demonstrated a significant correlation between mHag HA-1, -2, -4 and -5 mismatch and GvHD (17).

We aimed at the biochemical characterisation of human mH antigens. Thereto, we made use of the immunopurification and biochemical techniques succesfully applied by Rammensee and

15   his colleagues (18, 19) to extract murine mH peptides from MHC molecules. Indeed, HPLC separation of low Mr molecules (< kD) obtained from acid treated MHC class 1 HLA-A2.1 molecules appeared successful. Fractions with sensitizing activity for the non-sexlinked mh antigen HA-2 specific CTL clones were

20   isolated (20). To analyse the peptidic nature of the mHag HA-2, two sets of experiments were carried out. First, the sensitizing activity of the mHag containing fractions, obtained as described above, is susceptible to protease treatment; i.e. incubation of these mHag containing HPLC

25   fractions with pronase or proteinase K abolished the sensitizing activity (21). Second, the MHC encoded TAP1 and TAP2 gene products are required for mHag peptide presentation on the cell surface. The transporter genes TAP1 and TAP2

associated with antigen presentation are required for delivery
of peptides from the cytosol with the endoplasmic reticulum
(22). The availability of a human celline "T2") lacking both
transport and proteasome subunit genes enabled us to study the

5   processing and presentation of human mH antigens. We
demonstrated that the (rat) transport gene products TAP1 and
TAP2 were required for processing and presentation of
antigenic peptides from influenza virus and from the
intracellular mH protein HA-2 (23).

10   However, until the present invention no one has succeeded
in identifying amino acid sequences of antigenic peptides
relevant in the mHag system, nor has anyone succeeded in the
identification of the proteins from which they are derived.
We have now for the first time identified a peptide which is a

15   relevant part of mHag HA-2.

          Thus this invention provides a (poly)peptide comprising a
T-cell epitope obtainable from the minor Histocompatibility
antigen HA-2 comprising the sequence YXGEVXVSV or a derivative
thereof having similar immunological properties, wherein X

20   represents a leucine or an isoleucine residue.

          The way these sequences are obtained is described in the
experimental part. An important part of this novel method of
arriving at said sequences is the purification and the choice
of the starting material. Said novel method is therefor also

25   part of the scope of this invention. However, now that the
sequence is known, it is of course no longer necessary to
follow that method, because the peptides can easily be made
synthetically, as is well known in the art. Since routine

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techniques are available for producing synthetic peptides, it

is also within the skill of the art to arrive at analogs or

derivatives of the explicitly described peptides, which

analogs and/or derivatives may have the same or at least

5   similar properties and or activity. On the other hand analogs

which counteract the activity of the explicitly described

peptides are also within the skill of the art, given the

teaching of the present invention. Therefor derivatives and/or

analogs, be it of the same or different length, be it agonist

10   or antagonist, be it peptide-like or peptidomimetic, are part

of the scope of this invention.

A preferred embodiment of the present invention is the peptide

with the sequence YIGEVLVSV which induces lysis of the cell

presenting it at a very low concentration of peptide present.

15   This does not imply that peptides inducing lysis at higher

concentrations are not suitable. This will for a large part

depend on the application and on other properties of the

peptides, which were not all testable within the scope of the

present invention.

20   The peptides and other molecules according to the invention

find their utility in that they may be used to induce

tolerance of the donor immune system in HA-2 negative donors,

so that residual peripheral blood lymphocytes in the

eventually transplanted organ or the bone marrow, as it may be

25   does not respond to host HA-2 material in a HA-2 positive

recipient. In this way GvHD may be prevented. On the other

hand tolerance may be induced in HA-2 negative recipients in

basically the same way, so that upon receipt of an organ or

bone marrow from an HA-2 positive donor no rejection on the
basis of the HA-2 material occurs. It may be the case that the
HA-2 peptide acts in a non-allelic restricted manner. In that
case the tolerance induction is not restricted to HA-2
5   negative individuals.

For tolerance induction very small doses can be given
repeatedly, for instance intravenously, but other routes of
administration may very well be suitable too. Another
possibility is the repeated oral administration of high doses
10   of the peptides. The peptides may be given alone, or in
combination with othe peptides, or as part of larger
molecules, or coupled to carrier materials in any suitable
excipients.

Further applications of the peptide or derivatives
15   thereof lie in the prophylactic administration of such to
transplanted individuals to prevent GvHD. This can be done
with either agonists, possibly in combination with an
adjuvant, or with antagonists which may block the responsible
cells. This can be done with or without the concomittant
20   administration of cytokines.
Furthermore the peptides according to the invention can be
used to prepare therapeutic agents capable of eliminating a
subset of cells, directly or indirectly, especially cells of
hematopoietic origin. This can be illustrated by the following
25   examples, which refer to leukemia related therapeutic agents.
a)   A HA-2 positive recipient (in bone marrow
transplantation) can be subjected to an additional pre bone
marrow transplant conditioning regime. This means that an

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agent which specifically recognizes a peptide according to the invention (a HA-2 peptide) as presented on hematopoietic cells, which agent induces elimination of the cells presenting said peptide, is administered to the recipient before

5   transplantation. This agent will eliminate all residual cells (leukemic cells) of hematopoietic origin. Such agent include but are not limited to T cells (preferably provided with a suicide gene) and/or antibodies coupled to toxic moieties.

b)   A HA-2 negative donor for Bone marrow transplantation can

10   be vaccinated with a peptide according to the invention, a HA-2 peptide. Upon transplantation to a HA-2 positive recipient, the donor's immune system can eliminate any residual or recurrent HA-2 peptide presenting cells in the recipient which are of course leukemic.

15   c)   A transplanted HA-2 positive recipient, transplanted with HA-2 negative (or for that matter HA-2 positive) bone marrow and suffering from recurrent disease (relapse), i.e. HA-2 positive leukemic cells, can be treated with (again) an agent which specifically recognizes a peptide according to the

20   invention (a HA-2 peptide) as presented on hematopoietic cells, which agent induces elimination of the cells presenting said peptide. In case of HA-2 positive bone marrow being transplanted to the HA-2 positive recipient, it is still essential (in case of recurrent disease) to eliminate all HA-2

25   positive cells even though this includes the transplanted material, because otherwise the HA-2 positive leukemia will kill the recipient. In the latter case the patient can be retransplanted, if necessary.

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Diagnostic applications are clearly within the skill of the art. They include, but are not limited to HA-2 typing, detection of genetic aberrancies and the like.

Other therapeutical applications of the peptide include the induction of tolerance to HA-2 proteins in HA-2 related (auto)immune diseases, such as possibly in Rheumatoid arthritis. On the other hand they may be used in vaccines in HA-2 related (auto)immune diseases.

On the basis of the peptide described herein genetic probes can be produced which can be used to screen for the gene encoding the protein. On the other hand such probes may be useful in detection kits as well. On the basis of the peptide described herein anti-idiotypic B cells and/or T cells and antibodies can be produced. All these embodiments have been made possible by the present disclosure and therefor are part of the present invention.

The techniques to produce these embodiments are all within the skill of the art.

Dose ranges of peptides and antibodies and/or other molecules according to the invention to be used in the therapeutical applications as described hereinbefore are usually designed on the basis of rising dose studies in the clinic. The doses for peptides may lie between about 0.1 and 1000 μg per kg bodyweight, preferably between about 1 and 10 μg per kg bodyweight.

The invention will be described in more explanatory detail in the following experimental part.

10

EXPERIMENTAL

Using mHag specific CTL clones as in vitro tools, some murine and human mHag have been isolated from MHC molecules by

5   acid elution and were shown to be peptides presented by MHC molecules (13,14). Further characterization i.e. the exact amino acid sequence of the mHag peptides and the identification of the proteins from which these mHag originate, have sofar not been reported. Only a small number

10 of 'non-conventional defined' murine mHag, like the H-3 encoded β2 microglobulin alleles (15) and the Hmt restricted mitochondrial encoded maternally transmitted antigen (16), have been characterized. Here we report the identification, by tandem mass spectrometry, of the HLA-A2.1 restricted mHag HA-2

15 epitope.

To isolate the mHag HA-2, HLA-A2.1 molecules were purified by affinity chromatography from HLA-A2.1 positive Epstein Barr Virus (EBV)-transformed B lymphocytes (EBV-BLCL) expressing HA-2. The HLA-A2.1 bound peptides were isolated by

20 acid treatment followed by 10kD filtration (14). These low molecular mass molecules were fractionated by reverse phase HPLC and individual fractions were analyzed for mHag HA-2 sensitizing activity by incubation with the mHag HA-2 negative, HLA-A2.1 positive lymphoblastoid cell-line T2 in a

25 ^{51}Cr release assay. One fraction (fraction 33) sensitized T2 for lysis by the HA-2 specific CTL clone 5H17 (17) (Figure 1a). When this fraction was rechromatographed using a shallower gradient, HA-2 sensitizing activity was observed in

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fractions 37 and 38 (Figure 1b). However, as assessed using

microcapillary HPLC/electrospray ionisation tandem mass

spectrometry, the latter fractions still contained over 100

different HLA-A2 binding peptides (18). To determine which of

5   the peptides was responsible for the HA-2 sensitizing

activity, fraction 37 was analyzed using an on-line splitter

(19), allowing comparison of the mass spectrometric data with

results of the functional assay. Figure 2a shows a single peak

of HA-2 sensitizing activity in four adjacent wells. From the

10   many peptides present in these wells, the relative ion

abundance profile of four peptides (with mass to charge ratios

(m/z) of 651, 869, 979, 1000) matched the activity profile of

the HA-2 specific CTL activity. Collision activated

dissociation (CAD) analysis performed for the species with m/z

15   979 revealed the existence of 2 different peptides, YXGEVXVSV

and SXDFGTXQV (figure 3a and 3b). The X stands for L or I,

which cannot be distinguished by mass spectrometry under these

conditions. Synthetic peptide mixtures were made with an

equimolar mixture of L and I in place of X and assayed for

20   HA-2 specific sensitizing CTL activity. Only incubation with

peptide mixture YXGEVXVSV resulted in lysis of T2 (20).

In order to further define the natural processed peptide,

four single peptides were synthesized with I or L at positions

two and six and microcapillary HPLC coelution studies of these

25   synthetic peptides and the isolated fraction were performed.

Peptide YIGEVIVSV did not coelute with the natural processed

peptide and can therefore be excluded as the natural processed

epitope, whereas the other three peptides, YIGEVLVSV,

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YLGEVLVSV and YLGEVIVSV did coelute (21). These three peptides

all sensitized the T2 cell line for lysis by clone 5H17

(Figure 4a). Peptide YIGEVLVSV induced 50% lysis at a

concentration of 40 pM, whereas these concentrations were

5   substantially higher for peptides YLGEVLVSV and YLGEVIVSV

(1.5 nM and 2.25 nM). All three concentrations are within the

range of 10 pM-50 nM established for other naturally processed

epitopes (19,22). Clone 5H13 is an independently derived CTL

that, based on panel analysis, also recognizes HA-2, but

10   differs slightly in its fine specificity of antigen

recognition from 5H17 (10,23). Clone 5H13 also recognized all

3 peptide variants (Figure 4b). While the concentration of

peptides necessary to give half-maximal epitope reconstitution

were 5-10 fold higher than for 5H17, peptide YIGEVLVSV still

15   sensitized at 100 fold lower concentrations than the other

two. These results establish that, despite their fine

specificity differences (10,23), both HA-2 specific CTL

recognize the same peptide epitope.

Binding studies with these three peptides showed that

20   peptide YIGEVLVSV was the highest binder to HLA-A2.1. The

concentration that resulted in 50% inhibition of the binding

of the iodinated standard peptide to purified HLA-A2.1 was

5.6 nM, while those for YLGEVIVSV and YLGEVLVSV were 9.5 and

15 nM respectively (Figure 5). These values place these

25   peptides among the highest affinity naturally processed

peptides that have been defined so far (24). However, the

differences in binding affinities for these three peptides is

merely a factor of 3. The fact that YIGEVLVSV sensitizes for

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recognition by clones 5H17 and 5H13 at 50-100 fold lower
concentrations than the other two peptides indicates that this
peptide is recognized with highest affinity by the TCR and
thus may be the actual HA-2 epitope.

5   A search of DNA and protein sequence databases led to two
human sequences that both matched at 7 out of 9 residues to
peptide YIGEVLVSV. Peptide YYGEVCVSV is derived from
oligodendrocyte myelin glycoprotein (25) and peptide YIGSVLISV
was from unconventional myosin IC (26). Both human peptides
10   were synthesized and tested for sensitizing activity. Only the
myosin IC derived peptide YIGSVLISV could sensitize T2 cells
for lysis by 5H17 and 5H13 with a 50% lysis inducing
concentration of 5-50 nM (27). Human unconvential myosin IC
belongs to a large family of myosin genes (28,29), that
15   consist of different classes and that are indicated to be
involved in cell locomotion and organelle transport (28,29).
All cell types probably express several myosins from each
class simultaneously. Tissue restricted distribution of some
myosins has been reported (26,29). Database searches showed
20   that in different class I myosins of various origin, ranging
from *Acanthamoeba castellanii* to *human*, this peptide sequence
showed conservation for Y, I, G, V, and V at position 1, 2, 3,
5 and 9. Notably, the HA-2 peptide sequence differs in the
nonconserved amino acid positions from the myosin IC peptide
25   sequence. Human unconventional myosin IC is the only cloned
human class I myosin gene, but there is evidence for the
presence of at least 2 other class I myosins in human cells.
Therefore, it is not unlikely that an as yet unknown class I

myosin protein containing YIGEVLVSV is present in humans.
Interestingly, ongoing studies demonstrate the evolutionary
conservation of several mHag, including HA-2, between human
and non-human primates (30). Because mHag HA-2 is only

5   presented by haematopoietic cells, this unknown class I myosin
is either restricted to haematopoietic cells or is only
presented by haematopoietic cells because of tissue specific
processing.

       The polymorphism of mHag HA-2 is an intriguing issue. 95%

10   of the HLA-A2.1 positive population expresses the HA-2.
Consequently, the HA-2 specific CTL were generated in vivo
between a mHag HA-2 disparate bone marrow donor/recipient
combination. The HA-2 polymorphism can be explained by either
mutations in or adjacent to the HA-2 gene or by polymorphism

15   of the antigen processing system.
Until now, information on mHag has been extremely scarce.
Although the physiological function of mHag is still unknown,
their pivotal role in organ transplantation in general, and in
bone marrow transplantation in particular, is undeniable. We

20   herewith report, to our knowledge for the first time, the
amino acid sequence of a mHag defined by GvHD-derived CTL. The
availability of the mHag peptide sequence may allow in vivo
modification of the GvHD related T cell responses.
Furthermore, since mHag HA-2 is expressed on cells of the

25   hematopoietic lineage including leukemic cells, it is a
candidate for immunotherapy of leukemia prior to bone marrow
transplantation.

Fig. 5.   Binding of synthetic peptides to purified HLA-A2.1.
HPLC purified peptides were assayed for the ability to inhibit
the binding of iodinated hepatitis B core antigen peptide.
FLPSDYFPSV, to purified HLA-A2.1 molecules as previously
5   described (23). (closed circles), YIGEVLVSV; (closed
triangles), YLGEVLVSV; (closed squares), YLGEVIVSV; (closed
diamonds), the influenza M1 protein antigen GILGFVFTL. All
data points are the average of at least two independent
experiments.

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CLAIMS

1. A peptide constituting a T-cell epitope obtainable from the minor Histocompatibility antigen HA-2 comprising the sequence YXGEVXVSV or a derivative thereof having similar immunological properties, wherein X represents a leucine or an
5 isoleucine residue.

2. An immunogenic polypeptide obtainable from the minor Histocompatibility antigen HA-2 comprising the sequence YXGEVXVSV or a derivative thereof, wherein X represents a leucine or an isoleucine residue.

10 3. A peptide or polypeptide according to claim 1 or 2, comprising the sequence YIGEVLVSV.

4. A peptide or polypeptide according to claim 1 or 2, comprising the sequence YLGEVLVSV or YLGEVIVSV.

5. Vaccine comprising an epitope or a polypeptide according
15 to any one of claims 1-4.

5. A pharmaceutical formulation comprising an epitope or a polypeptide according to any one of claims 1-4.

6. Peptide or polypeptide according to claims 1-4 for use as a medicine.

20 7. Use of a peptide or polypeptide according to claims 1-4 in the preparation of a medicament for the induction of tolerance for transplants to prevent rejection and/or Graft versus Host disease.

8. A method for the elimination of a group of (neoplastic)
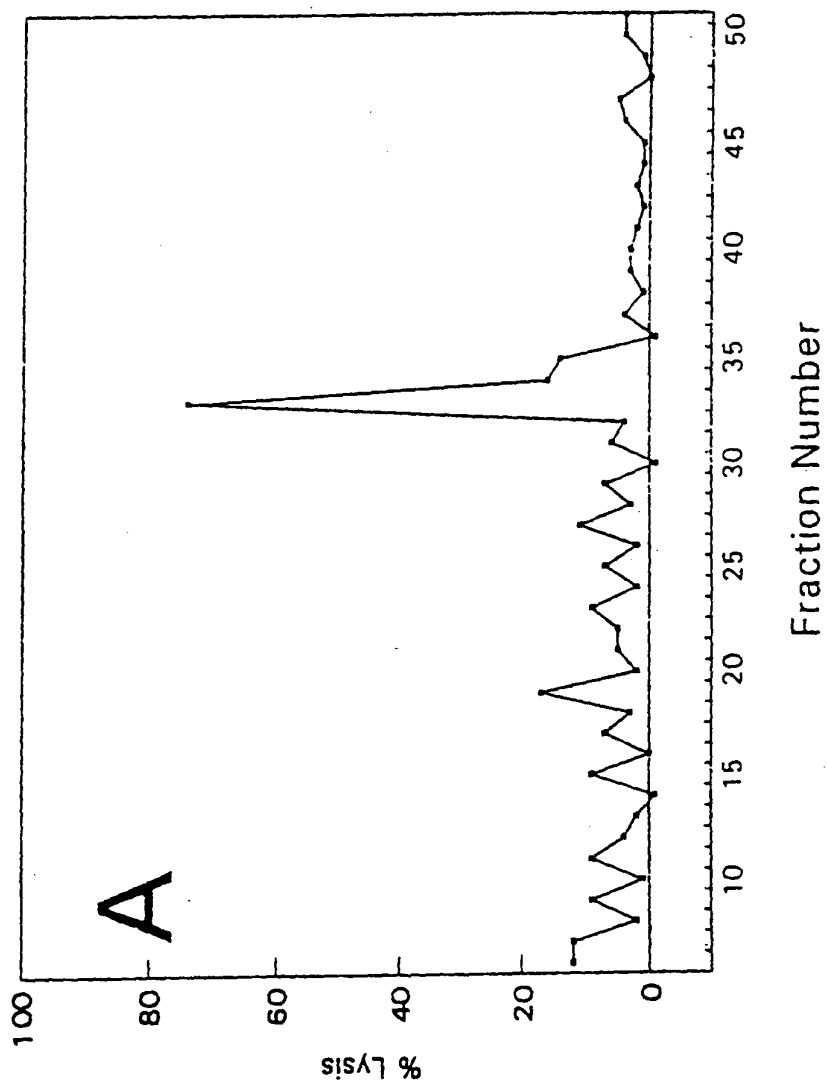25 hematopoietic cells presenting a peptide in the context of HLA

class 1 according to any of one of claims 1-4, whereby elimination is induced directly of indirectly by specific recognition of said peptide in said context.

9.   Analog of the peptide according to claim 1, which is an antagonist for the activity of T cells recognizing said peptide.

10.   Method for the generation of antibodies, T cell receptors, anti-idiotypic B-cells or T-cells, comprising the step of immunization of a mammal with a peptide or a polypeptide according to claim 1 or 2.

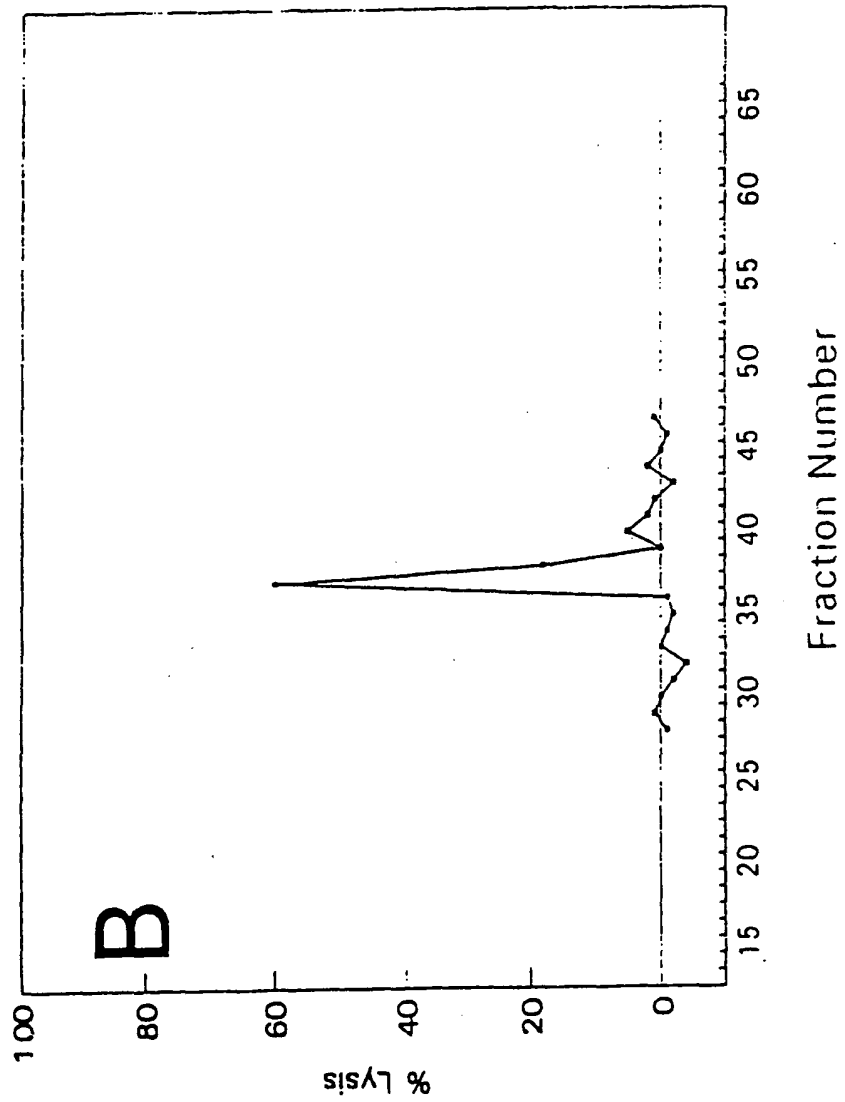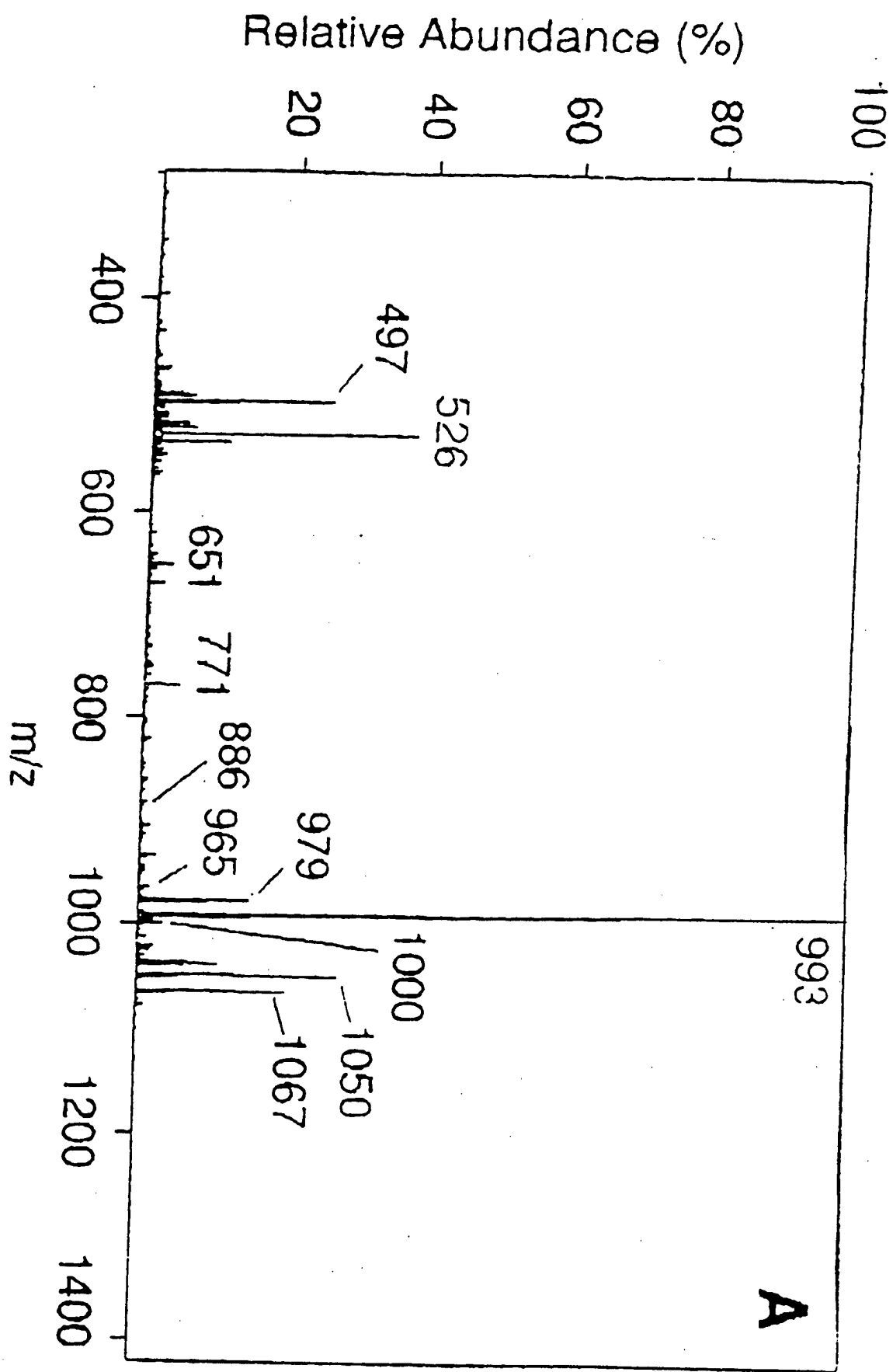11.   Antibodies, T-cell receptors, B-cells or T-cells obtainable by the method of claim 9.

A
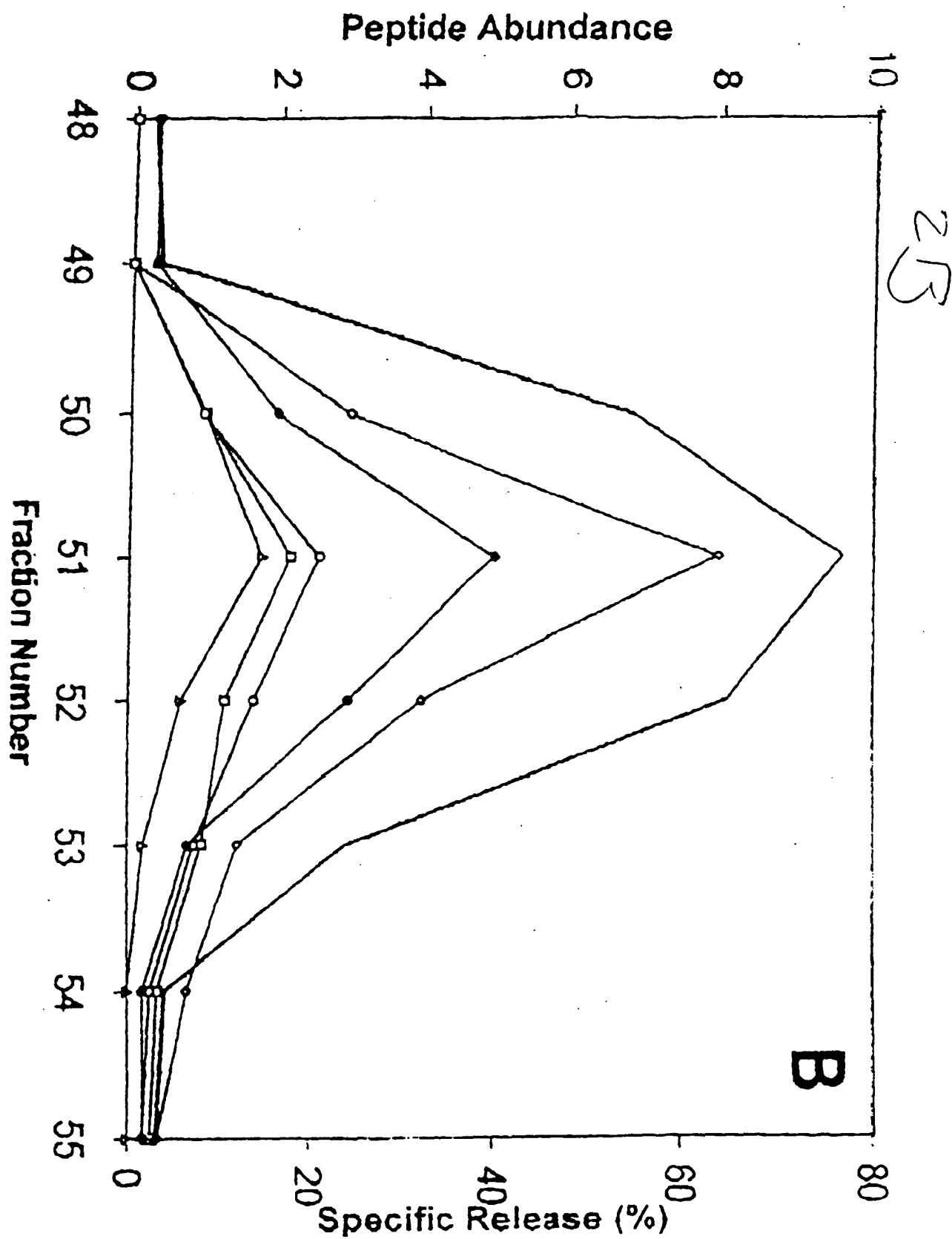
Fig. 1A

Fig 2B

3 A